

(12) UK Patent Application (19) GB (11) 2 273 772 (13) A

(43) Date of A Publication 29.06.1994

(21) Application No 9226229.4

(22) Date of Filing 16.12.1992

(71) Applicant(s)
Granta Laboratories Limited

(Incorporated in the United Kingdom)

123 Cambridge Science Park, Milton Road,
CAMBRIDGE, CB4 4FZ, United Kingdom

(72) Inventor(s)
Stanley Lucian Bogdanski
Stefan Nowinski

(74) Agent and/or Address for Service
Lloyd Wise, Tregear & Co
Norman House, 105-109 Strand, LONDON, WC2R 0AE,
United Kingdom

(51) INT CL⁵
G01N 33/543 21/17

(52) UK CL (Edition M.)
G1B BAG B108 B306 B307 B309 B508
G1A AA4 ACA AG18 AR7 AT3 AT5

(56) Documents Cited
EP 0246846 A2 JP 600224041 A

(58) Field of Search
UK CL (Edition L.) G1A ACA, G1B BAG BAX
INT CL⁵ G01N
ONLINE DATABASES: WPI, BIOTECH (DIALOG)

(54) Detection of macromolecules utilising light diffraction

(57) A probe is provided comprising a former of predetermined small size and selected geometry capable of diffracting light of a selected wavelength to produce a diffraction pattern.

The former has an active surface with reagent molecules exposed for binding the macromolecules to the active surface.

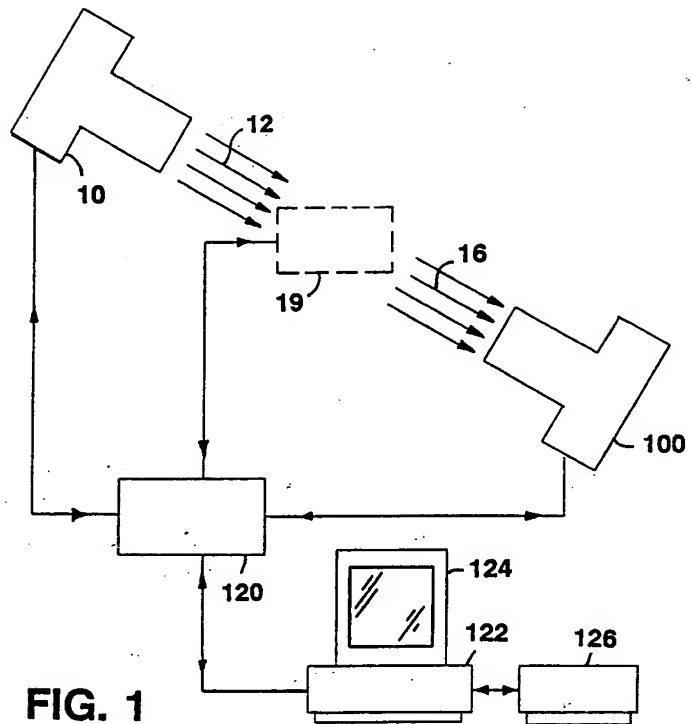
The wavelength of the light and the small size of the former are selected so that the macromolecules when bound to the active surface are non-transparent and produce a diffraction pattern change which is detectable and greatly amplified in dimension relative to the size of the macromolecule that produces such change.

A fluid containing the macromolecules is introduced to the active surface to enable binding of the macromolecules. Light of the selected wavelength is directed at the probe to produce the diffraction pattern and the diffraction pattern is detected and analyzed to indicate the presence of the macromolecules on the basis of change in the diffraction pattern.

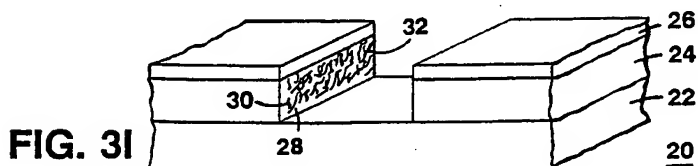
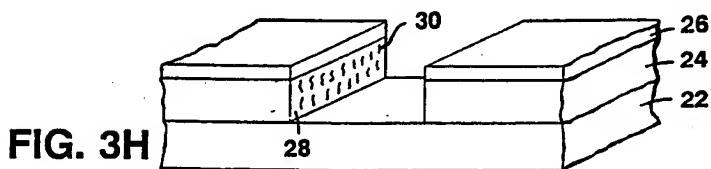
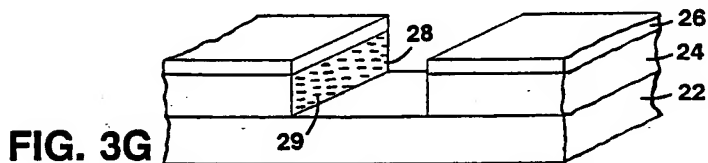
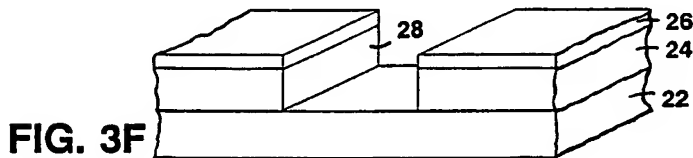
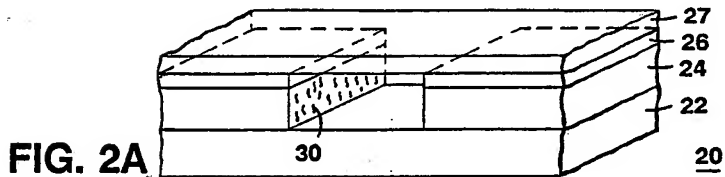
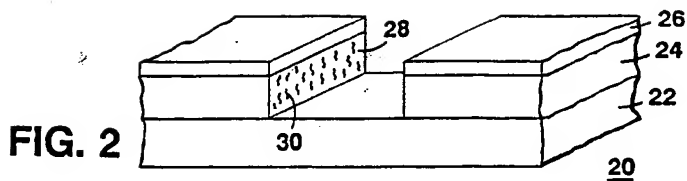
At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

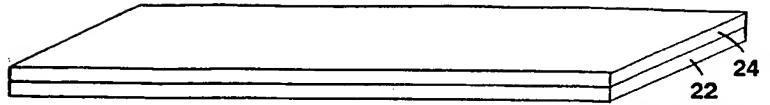
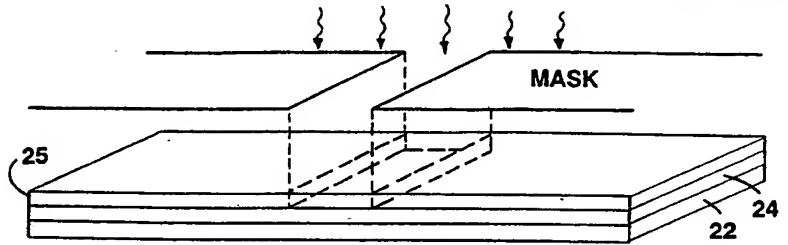
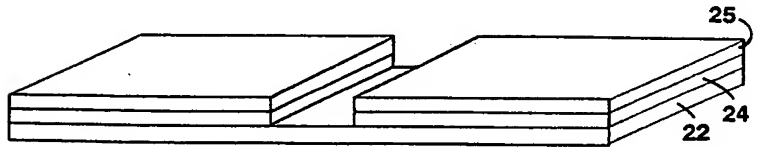
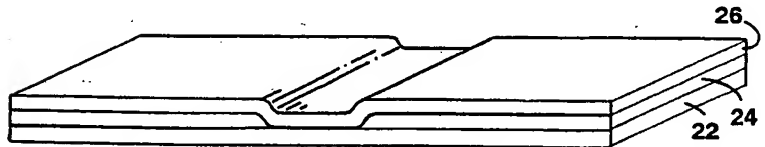
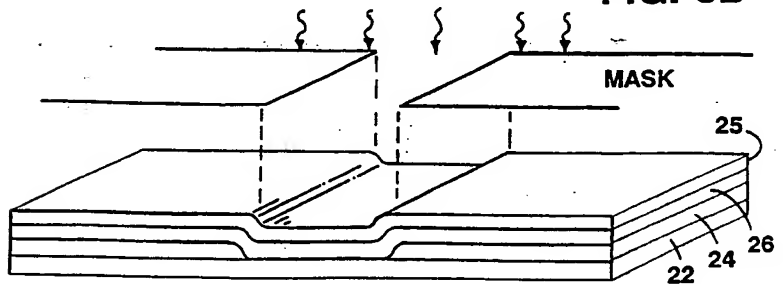
This print takes account of replacement documents submitted after the date of filing to enable the application to comply with the formal requirements of the Patents Rules 1990.

GB 2 273 772 A



2/9



**FIG. 3A****FIG. 3B****FIG. 3C****FIG. 3D****FIG. 3E**

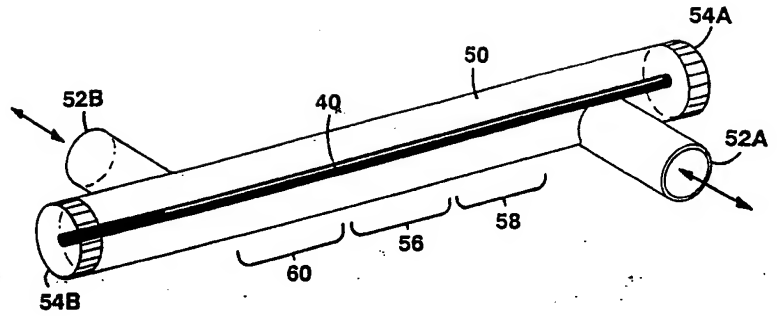


FIG. 4

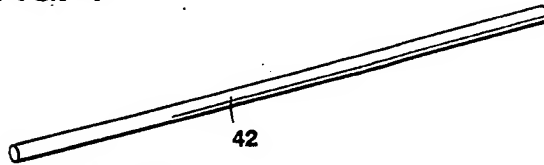


FIG. 5A

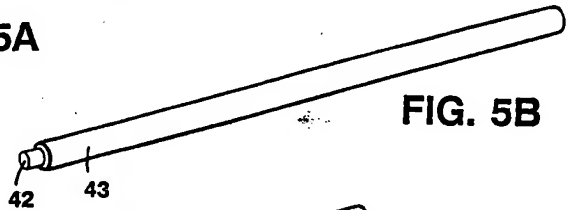


FIG. 5B

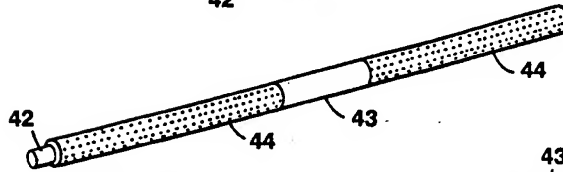


FIG. 5C

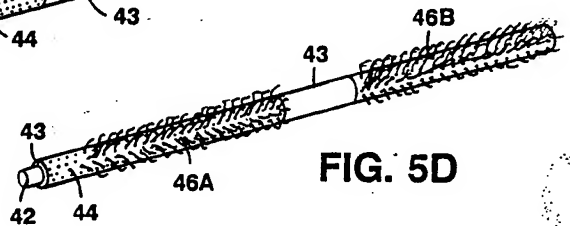
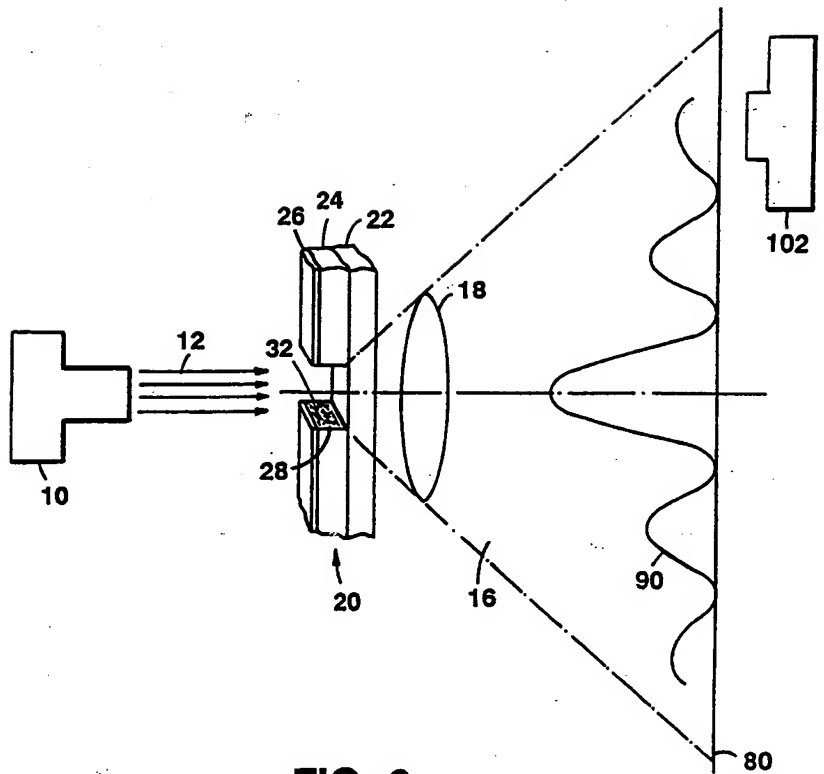


FIG. 5D

**FIG. 6**

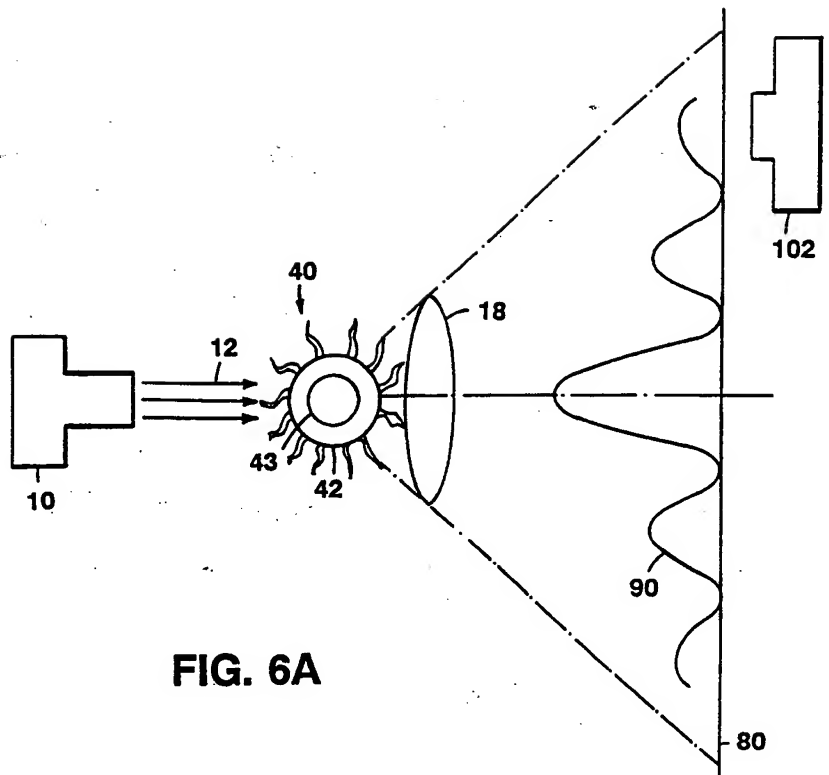
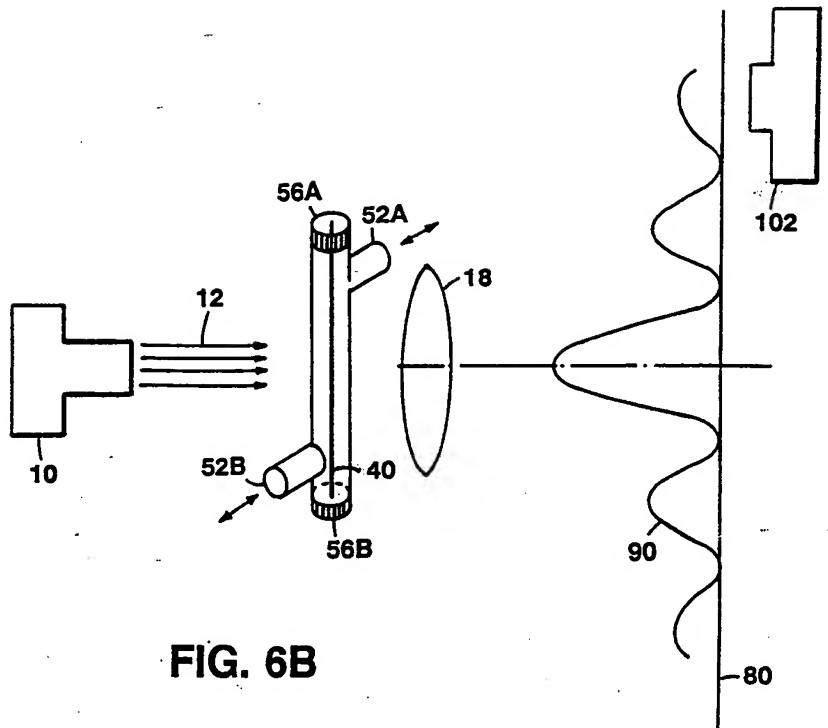


FIG. 6A



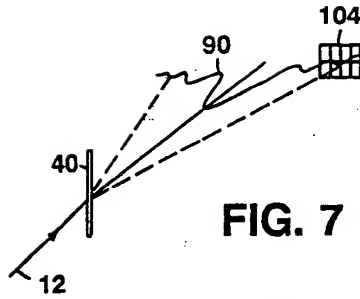


FIG. 7

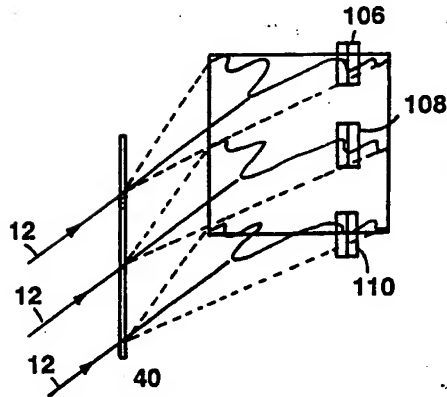


FIG. 8

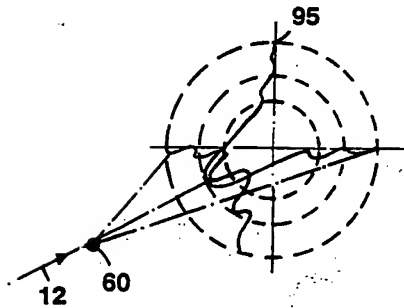


FIG. 9

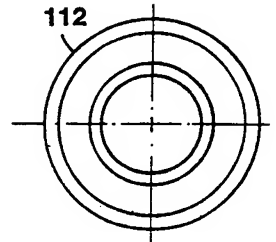


FIG. 9A

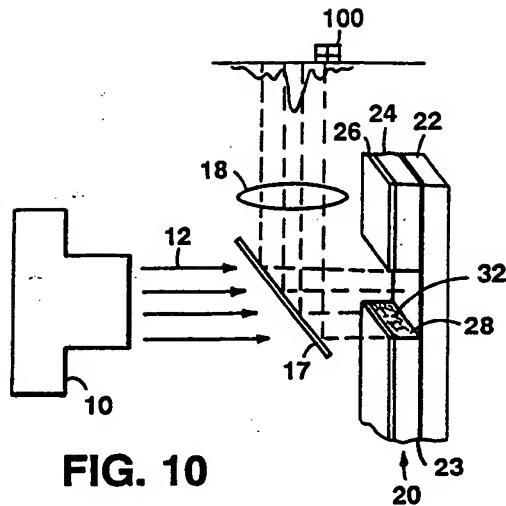


FIG. 10

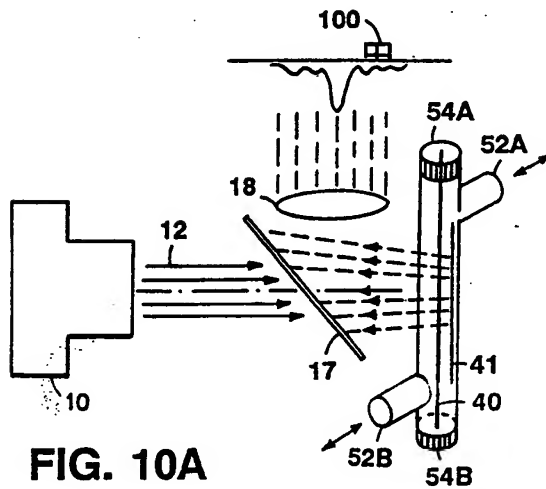


FIG. 10A

DETECTION OF MACROMOLECULES

This invention relates to the detection of macromolecules.

There are many highly selective and sensitive analytical methods for the detection of macromolecules of a biological interest. The selectivity of the method, in many cases, is based on the complementary structure which exists between certain pairs of molecules, such as:

a DNA or RNA single strand molecule and its complementary single strand,

an antibody and its antigen, and

an enzyme and its substrate (or coenzyme).

Essentially, it is these molecular interactions which give the analyses the ability to determine the presence of one particular molecule, the analyte, amidst many other compounds of a similar type. The reagent used comprises the molecular fragment which associates itself to the analyte.

Enhanced sensitivity and an improved limit of detection is often achieved by use of a reagent labelled with a molecular tag. The label makes the reagent and, therefore, the analyte, highly detectable by rendering it fluorescent, bioluminescent, chemiluminescent, radioluminescent, colourimetric, etc. A further technique for labelling, is by attaching an enzyme to the reagent and measuring the changes brought on by the introduction of the appropriate substrate.

Although detection limits as low as 600 molecules of analyte have been reported, immunoassays which are normally used can detect down to 10^{-14} mol/l of analyte, which is sufficient for many applications involving drugs, metabolites or gene products. Infection and infectious agents, as well as food contamination are two areas which benefit from the lowering of the limit of detection.

One way of improving the detection limit is to increase the number of analyte molecules in the sample. Culturing

samples is the most common approach at this time, but the use of polymerase chain reaction (PCR) and similar techniques applicable to DNA are expanding. Amplification techniques often result in multiplying the interfering substances along with the analyte.

Assay formats are classed as either heterogeneous or homogeneous, depending on whether a reagent is immobilized on a solid or whether it remains in a liquid, respectively. Assays are also classified as to whether they are competitive or non-competitive.

In a competitive, heterogeneous assay, there are two reagents. The first, R1 is selective towards the analyte, A, and is immobilized say on the surface of a microtitre plate. The sample containing the analyte is introduced and then washed away, leaving some of the R1 occupied by the analyte. A second reagent, R2 which is labelled and selective for the first reagent, R1, is added and washed away. The response of the detector towards the labelled reagent is indirectly proportional to the amount of analyte originally present.

In a non-competitive, heterogeneous assay two reagents are used. The first is immobilized and selective to the analyte. The analyte is captured, as described above. The second reagent is labelled and selective to the analyte. The following detector signal is directly proportional to the concentration of the analyte.

Homogeneous techniques basically are the same as the above but rely on a measurable change occurring to labelled reagent when it becomes associated to an analyte. Homogeneous assays are mostly of the competitive type and are usually relatively simple to perform and automate but have limited sensitivity, i.e. 10^{-9} mol/l.

These assay formats which involve multiple steps, having two or more reagents, make the analysis more complex and more prone to errors and contamination. The use of labels on reagents often indicates another level of conditions must apply which may compromise those which result in the best reagent selectivity and reagent/analyte affinity. The labels and their detection are subject to a

range of potential interferences, which lead to inaccurate interpretation of the final results unless procedures are carefully followed in a clean environment. Because of these difficulties, many of the analytical methods which are developed within the research laboratory will never be used in general practice. Direct detection of the analyte or analyte/reagent offers the best means of reducing potential interferences and analysis complexity.

A range of new direct measurement techniques have recently been reported which are being developed by major companies. One company has recently launched an analytical instrument based on Surface Plasmon Resonance and others are developing techniques using the ellipsometric and interferometric detection of an evanescent wave. Other investigations are looking at the changes of the oscillation frequency of a piezo electric crystal and the alteration to a surface acoustic wave due to the presence of an associated analyte. In each of the above methods, the reagent is immobilized on the surface of interest. Although still in their infancy, these new techniques appear to be inherently expensive and lack sensitivity.

The present invention is relatively simple to implement, has great sensitivity and retains the advantage of a direct analytical measurement.

We describe below a heterogeneous method in which the unlabelled reagent molecule is attached to a small surface, configured so as to create a diffraction pattern when placed into a specially designed detector. Changes in this diffraction pattern indicate whether the reagent is associated with an analyte or whether it is unassociated, i.e., no analyte is present. The probe can be configured in several ways to achieve an amplified diffraction pattern and allow an analyte measurement, e.g., as a slit, as a wire, as a ball or as multiples of these discrete elements.

Our method allows detection of the analyte directly, without the necessity of further amplification or the use of

labelled reagents. This results in a direct analysis, less prone to interferences and contamination, optimised for selectivity, and offering a relatively simple analytical procedure. The technique is amenable to the use of reagent labels and additional reagents to further improve the sensitivity and limit of detection.

The probe concentrates the analyte from a relatively large volume of solution to a known specific small area, depending on its binding affinity between captured reagent on the probe and the analyte. We envision the use of detection systems other than diffraction, which take advantage of the enhanced signal resulting from this concentration step and localization of the analyte.

The detection technique includes multiple analyte detection, blank readings and tests for false positives and false negatives by incorporating the appropriate sections into the probe during the fabrication. This is accomplished without increasing the complexity of the analytical procedure.

In certain embodiments, the probe is configured to perform as a continuous detector, say on the eluent of a chromatographic column, or to serve as a consumable, for a preliminary, quick test to be performed by a doctor in his surgery. The probe giving a positive test result and which is presumed to contain the analyte can be sent to a clinical laboratory for further tests, including amplification, and final confirmation.

Accordingly, in one aspect, the invention features method and a system for detection of macromolecules that employs a probe comprising a former of predetermined small size and selected geometry capable of diffracting light of a selected wavelength to produce a diffraction pattern, the former having an active surface with reagent molecules exposed for binding the macromolecules to the active surface; a light source adapted to emit light of the selected wavelength, the radiation being directed to and extending along the active surface; the wavelength of the

light and the small size of probe being so related that the macromolecules when bound to the active surface are non-transparent and produce a diffraction pattern change which is detectable and greatly amplified in dimension relative to the size of the macromolecule that produces the change; a detector arranged to detect at least a portion the diffraction pattern; and a system control adapted to determine the presence of the macromolecules on the basis of the change of the diffraction pattern.

Preferred embodiments of this aspect of the invention may have one or more of the following features.

The limited size of the active surface of the probe is effective to concentrate the macromolecules of interest on its surface for detection.

The reagent molecules are either DNA or RNA molecules of a sequence complementary to the sequence of DNA or RNA molecules of interest, or protein molecules.

The protein molecules are either enzymes adapted to detect substrates or co-enzymes, or antibodies associated to an antigen of interest.

The critical dimension of the probes' active surface is of the order of 0.1 to 20 microns and the macromolecules being detected have dimension of the order of fractions of a nanometer to nanometers.

The probe has a wire former that includes an intermediate layer with attached reagent molecules.

The probe comprises a former that defines a slit further including an intermediate layer with attached reagent molecules at the edges of the slit.

The probe comprises a 0.1 to 20 micron size sphere former that includes an intermediate layer with attached reagent molecules.

The probe comprises a former that defines an aperture including an intermediate layer with attached reagent molecules at the edge of the aperture.

The reagent molecules are arranged in a manner to form detection and reference surfaces.

The detection and reference surfaces enable quantitative or qualitative detection of the macromolecules.

In another aspect the invention features an apparatus for the detection of macromolecules comprising a light source, a probe comprising a former of predetermined dimensions and a detector, all arranged in a geometry such that the detector observes the diffraction pattern of the probe, the former having an active surface of such dimension that its diffraction pattern at the detection plane is of the order of centimeters from 1st to 2nd order peaks, whereby changes on the active surface of the order of nanometers in the dimension of the probe result in movements of the order of millimeters in the position of the diffraction peaks allowing the attachment of macromolecules to the active surface of the probe to be detected.

Preferred embodiments of this aspect of the invention may have one or more of the following features. The radiation is modified (such as being polarized), to enhance the interaction of the radiation with the analyte macromolecules. The radiation is parallel and coherent to generate the optimum diffraction pattern.

The macromolecules are modified by staining or other process. The macromolecules are labeled by a radioactive, fluorescent, bioluminescent, chemiluminescent, electroluminescent, chromophoric or conductive element, or an enzyme.

The radiation making up the diffraction pattern is monochromatic, achieved, e.g., by use of a monochromatic source or a narrow band filter anywhere in the beam path prior to the detector.

The probe is of a "positive" shape such as a wire or a sphere, or a "negative" shape such as a slit or a pin-hole having at least one dimension of a suitably small magnitude so as to create a suitably large diffraction effect that results in magnification of surface changes.

In another aspect, the invention features a system and method of detection of macromolecules (as in diagnosis of

disease when the macromolecules are biological molecules of clinical significance) whereby a suitable reagent (biological examples include: single strand DNA, RNA antibodies, or other reagent molecule) is attached to an active surface of small dimensions and the probe is presented to a suitably prepared sample, the presence of analyte molecules in the sample resulting in binding of those molecules to the reagent molecules and producing a small dimensional change on the active surface and thereafter observing the probe, the presence of analyte molecules being thereby detected directly, without the use of complicated labelling procedures as used in some other diagnostic techniques.

The invention is hereinafter more particularly described by way of example only with reference to the accompanying drawings in which:

Fig. 1 is a diagrammatic view of an embodiment of analyzer system in accordance with the present invention;

Fig. 2 is one preferred embodiment of an analyte selective probe with an active surface located on a slit;

Fig. 2A is the analyte selective probe of Fig. 2 adapted for in situ detection;

Figs. 3A, 3B, 3C, 3D, 3E, 3F, 3G, 3H, and 3I show schematically a method of fabrication of the analyte selective probe of Fig. 2;

Fig. 4 is another preferred embodiment of the analyte selective probe with the active surface on a wire held within a tube which provides a flow through cell configuration for introducing a sample;

Figs. 5A, 5B, 5C, and 5D show fabrication of the analyte selective probe of Fig. 4;

Figs. 6, 6A, and 6B show a transmission mode embodiment of the analyzer system of Fig. 1;

Fig. 7 shows a diffraction pattern monitored by a split detector arrangement of the analyzer system;

Fig. 8 shows multiple diffraction patterns formed by several beams and monitored by multiple detectors of the

analyzer system;

Figs. 9 and 9A show a diffraction pattern obtained by a random orientation of the active surface, and an annular detector used to detect this pattern, respectively; and

Figs. 10 and 10A show the reflection mode embodiments using a slit probe and a wire probe, respectively.

Referring to Figs. 1 and 2, a system for detecting macromolecules includes a source 10 emitting electromagnetic radiation 12 that is directed to a probe 20, containing the sample and held in a holder 19 in line with a detector 100. An active surface 28 of probe 20 is aligned and interacts with the radiation. The substances present on active surface 28 alter the outgoing radiation 16 in a predictable manner which is detected by detector 100. The entire operation is controlled by a process control unit 120 that specifies the type of radiation to be emitted from source 10, controls location of probe 20 and defines the mode of operation and position of detector 100. A system operator interacts with control unit 120 via a computer 122 and a display unit 124. A printer/plotter 126 is used to generate a hard copy. Detection system 100 has several detectors that can be used simultaneously or interchangeably depending on the type of the probe used, wavelength of the detection beam and reference beams, the overall source-probe-detector arrangement and the mode of operation. Depending on the mode of operation and the wavelength of the source, the selected detector is a photomultiplier, photodiode, CCD array, or photoconductive detector. The invention uses several types of probe 20.

Referring in particular to Fig. 2, analyte-selective probe 20 includes a rigid transparent surface 22, a thin SiO_2 layer 24, and a nontransparent layer 26. A thin section of layers 24 and 26 is removed to form a transparent slit which is $0.1\text{ }\mu\text{m}$ to $20\text{ }\mu\text{m}$ wide and about 0.02 mm to 20 mm long. The exposed silicon dioxide surface 28 of the slit is chemically treated to introduce reactive sites ($-\text{NH}_2$) which are used to immobilize single strands of DNA having a

sequence of nucleic acids which form matching base pairs with the complementary nucleic acids found on the targeted analyte DNA molecule. Surface 28 has a well defined depth, surface roughness, and geometry and specific orientation with respect to incoming beam 12.

The 0.1 μ m to 20 μ m wide slit is fabricated by a series of photolithographic steps. Referring to Fig. 3A, probe 20 is formed by depositing thin layer 24 of SiO₂ by chemical vapor deposition (CVD), sputtering or other technique onto transparent substrate 22 (e.g., microscope slide lightly coated with thin gold layer to make inactive). The thickness of SiO₂ layer 24 is selected depending on the desired depth of active surface area 28. A photoresist 25 is deposited on top of the silicon dioxide layer 24. The first photolithographic (Fig. 3B) step defines the dimensions of the slit on the probe and starts the process of removing the desired section of SiO₂ layer 24 which exposes active surface 28. Photoresist 25 is exposed and developed to define the slit. In the area defined by the photolithographic mask (Fig. 3B), SiO₂ layer 24 is removed by anisotropic etching (or by ion milling) through the SiO₂ layer (Fig. 3C). Next, photoresist 25 is removed. The nontransmitting layer 26 that is opaque to radiation of selected wavelengths (e.g., a reflecting gold layer) is deposited onto layer 24. In the second step, photoresist 25 is again deposited, exposed and developed to define a slit that coincides with the removed section in the SiO₂ layer (Fig. 3E). Etching (or ion milling) is used again to create a transparent slit in nontransmitting layer 26 (Fig. 3F).

Alternatively, only one photolithographic step is used after deposition of both layers 24 and 26. Here, a photoresist is deposited on layer 26, exposed and developed to define the slit. The slit is created by ion milling or by combination of ion milling and anisotropic etching through layers 24 and 26.

The silicon dioxide surface 28 is activated for the attachment of a reagent molecule. When using single

strand DNA fragments as reagent molecules, the silicon dioxide surface is activated using a silylating agent to form activated ester silica 29, i.e., N-hydroxysuccinimidyl-silica located on surface 28 (Fig. 3G). The desired single strand DNA fragments are synthesized by standard techniques (e.g., using an Applied Biosystems Synthesizer). The fragments have an additional section with an aminoalkyl group at the 5'-end. The 5'-aminoalkyl-DNA is then coupled to an N-hydroxysuccinimidyl ester silica located on the activated edge surface 28 (Fig. 3H). This reaction gives a stable amide linkage between the DNA and the silica, as described by Solomon, et al. in Analytical Biochemistry 203, 58-69 (1992).

Probe 20 contains a long, thin slit that has the interior SiO_2 edge coated with analyte selective reagent e.g., single strand DNA fragments 30 of a particular, known sequence. The sequence is chosen to be complementary to the sequence of the DNA material, analyte of interest, the particular probe is designed to detect. The probe uses different nucleic acid sequences for detection of different analytes of DNA.

Next, active surface 28 of probe 20 is contacted with the analyte solution by immersion or introducing the

test solution to the probe. The analyte solution is prepared by lysing cells to release DNA material. The double strand DNA is dissociated into single strands by adjusting the conditions of temperature, salt concentration and/or pH. The strands are cut into suitable lengths using restriction enzymes. The analyte DNA molecule, the target, may be considerably larger than the associated DNA immobilized on the surface of the probe. The analyte DNA material is bound to the immobilized strands by controlling the conditions in the micro-environment of the slit. A washing step is used to remove potential interference molecules which are less strongly bound. In many cases, the selectivity of the probe is related to the number of associated base pairs being formed between the probe DNA and the analyte DNA, whereas the limit of detection is more closely related to the size of analyte molecule and depth of the active surface layer containing the probe DNA.

In another embodiment, we use a wire probe, as shown in Fig. 4. Wire probe 40 is mounted in a capillary tube 50 using mounting ends 54A and 54B. The sample and subsequent wash solutions are introduced to the probe via ports 52A and 52B.

Referring to Fig. 5A, wire probe 40 comprises a former 42 of 0.1 to 20 microns in diameter (e.g., tungsten wire). The former is coated with a thin layer of silicon dioxide 43 introduced by chemical vapor deposition (Fig. 5B). Referring to Fig. 5C, selected areas of silicon dioxide layer are then activated using a silylating compound to form an activated ester such as N-hydroxysuccinimidyl-silica 44. The desired single strand DNA segments with a DNA sequence complementary to the sequence of a particular analyte are then immobilized on activated surfaces 44.

creating active surfaces 46A and 46B (Fig. 5D) in the same way as described for the slit probe.

In a similar manner, other sequences of DNA which are targeting other analytes are immobilized on different segments of the probe by a series of reaction steps which mask and reveal different portions of the probe. A multi-segment probe can be fabricated to indicate a false positive or false negative test result.

In another embodiment, the probe is configured to detect several analyte DNA molecules with a single exposure to the sample solution. On introduction of a test solution, all the above surfaces are contacted and subsequently washed under the same conditions. The system either examines the sections in series using a single channel detector or uses multi-channel detectors or scanning detectors. Thus several analytes can be detected simultaneously by the analyzer system; this is schematically shown in Fig. 8.

Alternatively, slit probe 20 designed for the multiple analyte detection includes similar sections as described above for the wire probe.

The light source 10 (e.g., an excimer laser, Helium-Neon laser, tunable YAG laser, etc.) emits continuous or pulsed monochromatic, collimated electromagnetic radiation of a suitable wavelength. Depending on the reagent and analyte being detected, the source is selected on the basis of wavelength from a range of ultra-violet, visible or infra-red lasers. Alternatively, light source 10 includes a source of white light. Suitable optics and filters are used to scan the wavelengths and direct the beam. The radiation is split into one or more detection beams and one or more reference beams. The geometry of the beam-active surface interaction is controlled. Material 26 surrounding the active surface of probe 30 is opaque to the radiation so that the plane of the active surface lies in the direction along the path of the radiation.

The wavelengths are selected on the basis of the active

surface, and probe configuration, spectral absorbance, reflectance or scatter characteristics of the reagent and analyte (suitable stains or other absorption enhancing techniques may be used) and the dimensional change as related to the presence of the analyte. The dimensional change, from fractions of a nm to few microns, is measured by monitoring the corresponding change in the diffraction pattern of the active surface with and without the analyte. The analyzer system can scan the active surfaces at different wavelengths and angles to obtain diffraction patterns with optimal signal-to-noise conditions.

Fig. 6 is a diagrammatic view of the transmission mode embodiment of the slit probe. Source 10 emits collimated monochromatic light 12. Probe 20 is aligned to have plane 28 of the active surface oriented along the path of the radiation. The diffracted light 16 is imaged by a spherical lens 18 onto an image plane 80 at a predetermined distance from the lens equal to its focal length. A detector 102 of detection system 100 placed in image plane 80 detects the far field (Fraunhofer) diffraction pattern 90 formed by the beam passing over the slit aperture containing active surface 28. Single element detector 102 scans the image plane and detects intensity and location of each peak of an interference pattern given by:

$$\frac{I(\theta)}{I(0)} = (1/\beta \sin \beta)^2$$

wherein θ is the angle from the axis of the straight through beam. Pattern 90 has a series of maxima and minima at distances from the axis of the straight through light which are inversely related to the width of the slit. Thus by detecting the change of spacing of the fringes of the diffraction pattern, with and without analyte, the system detects thickness of the analyte (and other layers) of active surface 28. The output of the detector is digitized and processed in the data analyzer of computer 122. Fig. 6A shows diagrammatically wire probe 40 placed in the analyzer system operating in the transmission mode embodiment. Fig.

6B shows diagrammatically an embodiment wherein wire probe 40 is used in a flow cell arrangement. In the flow cell arrangement, the test solution is recirculated or added to a flowing stream of cleansing solution.

Alternatively to scanning the diffraction image, multiple element detector 104, formed by several light detecting elements (e.g. photodiodes), is located at a fixed position. As shown in Fig. 7, detector 104 determines changes in the radiation intensity at a fixed point of a selected peak of diffraction pattern 90. From the change in the detected intensity, (i.e., movement of the fringe) presence and concentration of the analyte is determined. The size of the photosensitive surface area of the detector is optimized according to probe dimensions, distance between the detector and probe, and further optical considerations to give the best analyte resolution. The analyzer system determines, in real time, changes in the diffraction pattern that correspond to the active surface changes due to increasing presence of the analyte.

The above described configurations observe changes on the scale of sub-nanometers to nanometers using a slit of few microns. This resolution, which is dependent on the chemical and optical nature of the species present on active surfaces 28, is sufficient to detect the single strands DNA. Further reagents may be used to enhance the presence of the analyte and give a large detector response, e.g., by adding to the dimension of the analyte layer or making the analyte less transparent to the radiation from the source.

Referring to Fig. 8, in a multi-beam arrangement, multiple detectors 106, 108, and 110 are used in conjunction with several beams 12 to detect the diffraction patterns from different areas of the wire probe 40. This arrangement can analyze multiple analytes at the same time. Slit probe 20 may comprise several slits with active surfaces 28, each adapted to detect different species of analyte.

The high sensitivity is obtained from the small area of the probe onto which the analyte is concentrated, a high

affinity of binding between analyte and immobilized reagent, and a detector based on diffraction, which increases in amplification as the probe size is reduced. (Other direct methods for DNA analysis have signals which are proportional to surface area and, therefore, to number of analyte molecules.) Since a single probe can be fabricated with blank and reference sections as well as containing several captive reagents, a range of internal corrections are possible, e.g., to align the probe and eliminate false readings.

The applications of the probe/detector govern the operating conditions and the design of the probe. A simple "yes/no" qualitative test requiring maximum sensitivity benefits from using a probe with a high captive reagent concentration immobilized on a probe containing a minimum active surface area. The same conditions should be optimized to obtain maximum analyte/reagent affinity. A continuous detector may benefit from a larger active surface area, lower reagent concentration and sub-optimal conditions in order to obtain a quicker response time and avoid interferences accumulating over time.

The analyzer system utilizes one or more detection beams and one or more reference beams arranged to probe different active areas of the probe sequentially or simultaneously. The concentration of the attached single strands of DNA reagent is varied along the slit length periodically or continually. The detection beam may scan the active surface with varying concentration of the analyte bound to the reagent while the reference beam scans a corresponding active surface with varying concentration of the reagent. The obtained diffraction patterns are subtracted. The examination sequences are chosen to calibrate the probe, to eliminate false positive or negative readings, to determine concentration of one or more analytes in test solution.

The test solution is brought into contact with the probe in an open or closed system. Referring to Fig. 2A, a

closed system is achieved by adding a transparent layer 27 onto nontransmitting layer 36 to form a closed space. Then, a suitable micro-flow fluid handling system is attached to circulate fluid through the closed volume. The closed system enhances the control over temperature and pH parameters required to control the binding steps. The wire probe of Fig. 4 is used in the same way, wherein the closed system is achieved by connection of the micro-flow system to ports 52A and 52B. During the real time measurements binding of the analyte is detected by changes to the time base profile of the detected light.

The technique uses either a disposable probe or a probe that can be regenerated for multiple uses depending on the application. The multiple use probe is adapted to release the analyte from the active surface (e.g., by thermally cycling the probe or by pH cleansing).

In another embodiment, the active surface of slit probe 20 (or wire probe 40) includes multiplicity of surfaces with single strands of DNA arranged in a selected geometry that allow only molecules of a certain length to adhere. For example, there may be two active surfaces separated by a selected distance. Each surface has an immobilized reagent, and the selection of analyte is based on two different molecular associations having a defined distance (molecular size) between them.

In another transmission embodiment, the source-probe-detector alignment can be simplified by using several randomly positioned wires or micro-spheres as a former. For this arrangement, the diffraction pattern has the peaks located circularly. Referring to Fig. 9, beam 12 irradiates a suspension of spherical probes 60 (e.g., former is a 3 to 5 micron silica ball similar to the ones used in the solid phase chromatography and creates diffraction pattern 95 that is monitored by a detector 112. Detector 112 has the detector elements arranged in one or more annular rings centered on the straight through beam (Fig. 9A). Detector 112 is positioned at a distance from the axis that enables

proper resolution of the fringes. The greater the distance from the axis, the greater the displacement of the fringes.

Figs. 10, and 10A show diagrammatically the reflecting mode embodiment of the analyzer system, wherein the diffraction pattern is observed by detecting the reflected radiation from the probe. Referring to Fig. 10, the reflecting embodiment of slit probe 20 requires flat transparent substrate 22 made reflective by depositing an additional reflective layer 23 onto layer 22 prior to the slit formation. Reflective layer 23 reflects the diffracted radiation which is separated from the incoming beam by a half silvered mirror 17 at 45° or a similar optical element suitable for the selected wavelength. Detector system 100 is positioned to observe the reflected diffraction pattern in the same way as discussed above. Similarly, the reflecting embodiment of wire probe 40 (Fig. 10A) includes a mirror surface 41 positioned behind the wire. The incoming radiation, diffracted of the active surface of probe 40, is reflected back onto the wire and then detected by detector 100.

Optimization of detection may be achieved by altering the incidence angle of the radiation, by polarizing the radiation and rotating the polarized plane angle relative to the active surface, by adjusting the pulse parameters of radiation, or by modulating the frequency. Furthermore, the light source can operate in a pulse mode to prevent thermal effects on the active surface or to scan different regions of the active surface interchangingly. Each of these techniques provides a means of internal noise and drift reduction of the detector as well as that of the environment (e.g., vacuum, gas or liquid) wherein the measurement is conducted.

Other probe embodiments use only a former with the immobilized selective reagent bound directly to the former. Alternatively, a number of reactive intermediates selectively paired with reactive moieties can be used.

Reagent molecules can be arranged so as to coil and

grow more dense when affected by the analyte molecule, effectively changing the active surface. Immobilization occurring at several points between the reagent and active surface can ensure optimal positioning of molecules for their subsequent detection. The reagent molecules so arranged would respond to the angle of the plane using polarized radiation. This arrangement reduces noise and enhances the detection.

In another embodiment, the SiO_2 layer activated by a silylating agent is replaced by other "active" layers to which the preferred type DNA strands are bound, for example, polystyrene, nitro-cellulose, nylon. The detection surfaces are again fabricated by photolithography; however, other methods such as micromachining or molding may also be used.

We also envision using probes with different shapes of active surfaces arranged to selectively concentrate the analyte. An aperture with a 0.1 to 20 micron diameter resolves features down to 0.1 nm layers of analyte. The slit type apertures were described in detail; however, other geometries such as circular or square hole apertures may be also used. Furthermore, the active surfaces may be located on structures that are complementary to the described apertures. For example, the probe is an opaque long, thin bar or a dot with the activated surfaces located on the outside edges. An equivalent of a circular aperture is a small sphere.

Following the introduction of the sample and association between the probe and analyte, the diffraction signal can be augmented by enlarging the analyte layer or making it less transparent. This is accomplished by using reagents which would react or combine with the now immobilized analyte. These reagents could merely add to the size of the analyte layer, stain the analyte, or attach an enzyme (or reactive molecule) which would subsequently deposit a precipitate in this immediate vicinity. The probe can subsequently be also reacted with reagents which fluoresce, luminesce, etc.

CLAIMS

1. A method of detection of macromolecules comprising the steps of:

(a) providing a probe comprising a former of predetermined small size and selected geometry capable of diffracting light of a selected wavelength to produce a diffraction pattern, said former having an active surface with reagent molecules exposed for binding the macromolecules to said active surface;

(b) the wavelength of the light and the small size of the former being so related that the macromolecules when bound to said active surface are non-transparent and produce a diffraction pattern change which is detectable and greatly amplified in dimension relative to the size of the macromolecule that produces such change;

(c) introducing a fluid containing said macromolecules to said active surface to enable binding of the macromolecules;

(d) directing light of said selected wavelength at said probe to produce said diffraction pattern; and

(e) detecting and analyzing said diffraction pattern to detect the presence of the macromolecules on the basis of change in the diffraction pattern.

2. A method of detection of macromolecules comprising the steps of:

(a) providing a light source, a probe comprising a former of predetermined dimensions, and a detector, all arranged in a geometry such that the detector observes a diffraction pattern of the probe, the former having an active surface of such dimension that its diffraction pattern is of the order of centimeters from 1st to 2nd order peaks,

(b) introducing a fluid containing said macromolecules to the active surface to enable binding of said

macromolecules to reagent molecules located on the active surface, and

(c) detecting the attachment of said macromolecules to the active surface of the probe by determining changes in the position of the diffraction peaks of the order of millimeters corresponding to changes in the dimension of said active surface of the order of nanometers.

3. The method of claim 1 or 2 wherein the limited size of said former is effective to concentrate said macromolecules of interest on its surface for detection.

4. The method of claim 1, 2 or 3 wherein said reagent molecules are DNA molecules of a sequence complementary to the sequence of DNA macromolecules of interest.

5. The method of claim 1, 2 or 3 wherein said reagent molecules are protein molecules.

6. The method of claim 5 wherein said protein molecules are enzymes adapted to detect co-enzymes or substrates.

7. The method of claim 5 wherein said protein molecules are antibodies associated with an antigen of interest.

8. The method of claim 1, 2 or 3 wherein said reagent molecules are RNA molecules of a sequence complementary to the sequence of macromolecules of interest.

9. The method of any one of the above claims wherein the critical dimension of said former is of the order of 0.1 to 20 microns and the macromolecules being detected have dimension of the order of fractions of a nanometer to nanometers.

10. The method of any one of the above claims wherein said former is a wire that includes an intermediate layer with attached reagent molecules.

11. The method of any one of claims 1 through 9 wherein said former defines a slit in said probe, and said active surface further includes an intermediate layer with attached reagent molecules at the edges of the slit.

12. The method of any one of claims 1 through 9 wherein said probe comprises a 0.1 to 10 micron size sphere former that includes an intermediate layer with attached reagent molecules.

13. The method of any one of claims 1 through 9 wherein said former defines an aperture in said probe and said aperture includes an intermediate layer with attached reagent molecules at the edge of said aperture.

14. The method of any one of claims 10 through 13 wherein said reagent molecules are arranged in a manner to form detection and reference surfaces.

15. The method of any one of claims 10 through 13 wherein said detection and reference surfaces enable qualitative detection of said macromolecules.

16. The method of any one of claims 10 through 14 wherein said detection and reference surfaces enable quantitative detection of said macromolecules.

17. The method of any one of claims 1 through 16 wherein the light is modified to enhance its interaction with the analyte macromolecules.

18. The method of claim 17 wherein the radiation is parallel and coherent to generate the optimum diffraction

pattern.

19. The method of claim 35 wherein the radiation making up the diffraction pattern is monochromatic.

20. The method of any one of claims 1 through 16 and claim 32 wherein the macromolecules are modified, (such as staining).

21. The method of any one of claims 1 through 16 wherein the probe is of a "positive" shape such as a wire or a sphere, or a "negative" shape such as a slit or a pin-hole having, at least one dimension of a suitably small magnitude so as to create a suitably large diffraction effect and so magnification of surface changes.

22. A method of detection of biological macromolecules of clinical significance for diagnosis of disease utilizing a probe with a suitable reagent attached to an active surface of small dimensions and comprising the steps of presenting a suitably prepared sample to the probe, the presence of analyte molecules in the sample resulting in binding of those molecules to the reagent molecules and producing a small dimensional change on the active surface and thereafter observing the probe, detecting directly the presence of analyte molecules without the use of complicated labelling procedures as used in some other diagnostic techniques.

23. A method of detecting biological macromolecules from a solution, said method comprising the steps of:

(a) providing a probe comprising a former with an active surface having the critical dimension in the size range of 0.1 to 100 micron said active surface including reagent molecules exposed for binding the macromolecules of interest to said active surface;

(b) exposing said active surface to a solution

containing said macromolecules until they are attached, the limited size of the surface serving to highly concentrate said macromolecules relative to their concentration in the solution;

(c) providing a label capable of attaching to said macromolecules of interest, said label generating a detectable signal;

(d) detecting said signal using a detector of geometry related to the shape of said active surface; and

(e) analyzing said detected signal to determine the presence of said macromolecules.

24. The method of claim 23 wherein said label is a radioactive element.

25. The method of claim 23 wherein said label is a fluorescent element.

26. The method of claim 23 wherein said label is a enzyme element.

27. The method of claim 23 wherein said label is a bioluminescent element.

28. The method of claim 23 wherein said label is a chemiluminescent element.

29. The method of claim 23 wherein said label is a electroluminescent element.

30. The method of claim 23 wherein said label is a chromophoric element.

31. The method of claim 23 wherein said label is a electrical conductive element.

32. A system for detection of macromolecules

comprising:

a probe comprising former of predetermined small size and selected geometry capable of diffracting light of a selected wavelength to produce a diffraction pattern; said former having an active surface with reagent molecules exposed for binding the macromolecules to said active surface;

a light source adapted to emit light of said selected wavelength, said radiation being directed to and extending along said active surface;

said wavelength of the light and the small size of said former being so related that the macromolecules when bound to said active surface are non-transparent and produce a diffraction pattern change which is detectable and greatly amplified in dimension relative to the size of the macromolecule that produces the change;

a detector arranged to detect at least a portion said diffraction pattern; and

a system control adapted to determine the presence of said macromolecules on the basis of said change of the diffraction pattern.

33. The system of claim 32 wherein the limited size of the active surface of said probe is effective to concentrate said macromolecules of interest on its surface for detection.

34. The system of claim 32 or 33 wherein said reagent molecules are DNA molecules of a sequence complementary to the sequence of DNA macromolecules of interest.

35. The system of claim 32 or 33 wherein said reagent molecules are protein molecules.

36. The system of claim 35 wherein said protein molecules are enzymes adapted to detect substrates or co-enzymes.

37. The system of claim 32 or 33 wherein said reagent molecules are RNA molecules of a sequence complementary to the sequence of macromolecules of interest.

38. The system of claim 35 wherein said protein molecules are antibodies associated to antigen of interest.

39. The system of any one of claims 32 through 38 wherein the critical dimension of said former is of the order of 0.1 to 20 microns and the macromolecules being detected have dimension of the order of fractions of a nanometer to nanometers.

40. The system of any one of claims 32 through 39 wherein said former is a wire that includes an intermediate layer with attached reagent molecules.

41. The system of any one of claims 32 through 39 wherein said former defines a slit in said probe, and said active surface further includes an intermediate layer with attached reagent molecules at the edges of said slit.

42. The system of any of claims 32 through 39 wherein said probe comprises a 0.1 to 20 micron size former sphere that includes an intermediate layer with attached reagent molecules.

43. The system of claims 32 through 39 wherein said former defines an aperture in said probe and said aperture includes an intermediate layer with attached reagent molecules at the edge of said aperture.

44. The system of claims 40, 41, 42, or 43 wherein said reagent molecules are arranged in a manner to form detection and reference surfaces.

45. The system of claim 44 wherein said detection and

reference surfaces enable qualitative detection of said macromolecules.

46. The system of claim 44 wherein said detection and reference surfaces enable quantitative detection of said macromolecules.

47. A system for the detection of macromolecules including a light source, a probe comprising a former of predetermined dimensions, and a detector, all arranged in a geometry such that the detector observes the diffraction pattern of the probe, the former having an active surface of such dimension that its diffraction pattern at the detection plane is of the order of centimeters from 1st to 2nd order peaks, whereby changes on the active surface of the order of nanometers in the dimension result in movements of the order of millimeters in the position of the diffraction peaks allowing the attachment of macromolecules to the active surface of the probe to be detected.

48. The system of claim 47 wherein the radiation is modified to enhance the interaction of the radiation with the analyte macromolecules.

49. The system of claim 48 wherein the radiation is parallel and coherent to generate the optimum diffraction pattern.

50. The system of any one of claims 47 through 49 wherein the radiation making up the diffraction pattern is monochromatic.

51. The system of claim 47 wherein the macromolecules are modified.

52. The system of any one claims 47 through 51 wherein the probe is of a "positive" shape such as a wire or a sphere, or a "negative" shape such as a slit or a pin-hole

having at least one dimension of a suitably small magnitude so as to create a suitably large diffraction effect and so magnification of surface changes.

53. A system for detecting biological macromolecules from a solution comprising:

a probe having an active surface of limited size in the range of about 1 micron to 10 millimeters said active surface including reagent molecules exposed for binding the macromolecules of interest to said active surface;

a device adapted to introduce a solution containing said macromolecules of interest until they are attached, the limited size of said active surface serving to highly concentrate said macromolecules relative to their concentration in the solution; said macromolecules having a label capable to generate a detectable signal;

a detector of geometry correlated to the shape of said active surface adapted to detect said signal; and

a system control adapted to analyze said detected signal to determine the presence of said macromolecules.

54. The system of claim 53 wherein said label is a radioactive element.

55. The system of claim 53 wherein said label is a fluorescent element.

56. The system of claim 53 wherein said label is a enzyme element.

57. The system of claim 53 wherein said label is a bioluminescent element.

58. The system of claim 53 wherein said label is a chemiluminescent element.

59. The system of claim 53 wherein said label is a

electroluminescent element.

60. The system of claim 53 wherein said label is a chromophoric element.

61. The system of claim 53 wherein said label is a electrical conductive element.

Relevant Technical fields

(i) UK Cl (Edition L) G1B (BAG; BAX), G1A (ACA) *

(ii) Int Cl (Edition 5) G01N

Databases (see over)

(i) UK Patent Office

(ii) ONLINE DATABASES: WPI, BIOTECH (DIALOG)

Search Examiner

NICOLA CURTIS

Date of Search

10 MARCH 1993
ADDITIONAL SEARCH
MADE 21 MAY 1993

Documents considered relevant following a search in respect of claims

1-21, 32-52

Category (see over)	Identity of document and relevant passages	Relevant to claim(s)
X	EP 0246846 A2 (AMERSHAM INTERNATIONAL) - see page 1, line 21 - page 2 line 2 - page 3, line 25 - page 4 line 13 - page 7 line 6 - line 25	1,4-8, 17,32, 34-38
X	WPI ABSTRACT ACCESSION NO: 87-145708121 and JP 60224041 (TORAY INT INC) * Indicates additional entry	1,3-8,17, 18,32, 34-38

Category	Identity of document and relevant passages -30-	Relevant to claims.

Categories of documents

X: Document indicating lack of novelty or of inventive step.

Y: Document indicating lack of inventive step if combined with one or more other documents of the same category.

A: Document indicating technological background and/or state of the art.

P: Document published on or after the declared priority date but before the filing date of the present application.

E: Patent document published on or after, but with priority date earlier than, the filing date of the present application.

&: Member of the same patent family, corresponding document.

bas s: The UK Patent Office database comprises classified collections of GB, EP, WO and US patent specifications as outlined periodically in the Official Journal (Patents). The on-line databases considered for search are also listed periodically in the Official Journal (Patents).